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(57) Abstract

A method for the detection of a nucleotide sequence of a nucleic acid in a sample. The method comprises the steps: (i) contacting under hybridization condition the single stranded form of the nucleotide sequence with a single stranded nucleic acid probe, in which a plurality of rare earth metal chelate groups is covalently linked via a water-soluble polymer of non-nucleic acid structure to a nucleotide sequence complementary to the sequence to be detected, for the formation of a double stranded nucleic acid that as one of its strand has the nucleotide sequence to be detected and as the other strand the nucleotide sequence of the probe, and (ii) detecting the formation of double stranded nucleic acid containig the probe by measuring the time-resolved fluorescence from the rare earth chelates incorporated in said double stranded nucleic acid. The plurality of rare earth metal chelate groups have at least one metal ion selected from the group consisting of Eu^{3+}, Sm^{3+}, Tb^{3+} and Dy^{3+} as the chelated rare earth metal. The probes as given above are also claimed.

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HYBRIDIZATION ASSAY AND MEANS TO BE USED IN THE ASSAY

Background of the invention

This invention relates to a hybridization assay utilizing nucleic acid probes labelled with lanthanide chelates that show time-delayed fluorescence.

Labelled nucleic acids have become indispensable in hybridization assays, performed both in vitro and, as in hybridocytochemical microscopy, also in vivo. Appropriate labelling of nucleic acids is a crucial point in their sequencing and applications may also be found in the different methodologies of nucleic acid separation. Nowadays, most of the efforts to find more sensitive markers have been made in the field of hybridization probes for the detection of specific, complementary nucleic acid sequences. This is natural in view of the great importance of such assays in medicine and molecular biology.

DNA or RNA can be labelled in a variety of ways. Generally, all labels may be detected directly i.e. the label, which is bonded to the nucleic acid is itself detectable, or indirectly when the label participates in one or more reactions thus generating detectable products.

Description of prior art

Several new labelling methodologies have recently become available. Despite of many differences they can be systematized on the basis of some main criteria. In nucleic acid technology a common name for different labels is reporter group.

1. Methods of DNA detection

a) Direct detection method: Nucleic acids are commonly labelled with the radioisotopes ^{32}P, ^{125}I, ^{3}H or fluorescent markers. Especially in routine analyses the radioactive material tends to be replaced by the non-radioactive labels because of the serious drawbacks associated with the use of radioactive labels. Safety and disposal problems are obvious, but the low stability of materials with high specific activity together with their high cost should not be forgotten either.

Theoretically, fluorescent compounds are ideally suited to replace radioactive isotopes. To date the only examples of such fluorescent markers used in DNA labelling are fluorescein, rhodamine, Texas Red and NBD. The calculated high sensitivity which could be achieved using this type of reagents is, however, to a great extent limited by the fact that most biological samples including proteins also fluoresce thus bringing the background to a not always acceptable level.

b) Indirect detection method: Nucleic acids are labelled with different proteins possessing enzymatic activities e.g. alkaline phosphatase or horse radish peroxidase. The subsequent reaction of the appropriate substrate catalyzed by the attached enzyme usually generate an easy-to-detect coloured product. It is constantly emphasized that an advantage of such a system is the fact that there is no need for a detecting apparatus, but this fact can also be seen as a disadvantage since this visual technique is not well-suited to quantitative analysis.

2. Attachment of a detectable group to DNA or RNA

The way in which reporter groups are linked to nucleic acids may serve as another criteria of differentiation of the labelling techniques.

Direct attachment means that the detectable marker is bound to the nucleic acid already before a particular analytical process takes place. The label may be coupled to the nucleic acid enzymatically as in radiolabelling, by using DNA kinase and γ ^{32}P ATP, or by employing different α ^{32}P nucleoside triphosphates in the nick translation process. A properly activated label which is able to react with any existing or created function in the nucleic acids, can also be chemically attached to nucleic acids.

Indirect attachment of the detectable group to nucleic acids can be realized by several methods. Among the most commonly used ones is the labelling of nucleic acids with a hapten, thus rendering them detectable by immunological means.

Fluorescein has been proposed, and later also a trinitrophenyl group (TNP) as haptens, because antibodies with high affinity to these haptens were well-studied and easily obtainable. However, the complexity of nucleic acid derivatization together with a rather complex system of detection, problems in purification of some of the compounds, and quite a low sensitivity of the assay, made this technique rather unattractive.

N-acetoxy-N-2-acetaminofluorence (AAF) has been used for the chemical modification of nucleic acids for a sensitive, colorimetric detection of the target DNA using specific antibodies and peroxidase or alkaline phosphatase second antibody conjugates.

In view of the strong carcinogenic properties of AAF, other types of labels have been tried. Thus biotin was attached chemically to deoxyribonucleotides and these nucleotides were introduced into DNA by nick translation. A single stranded portion of biotin-DNA thus has been used as a hybridization probe. To detect double stranded DNA having biotin or hapten bound to one of its strands avidin or anti-hapten antibodies, respectively, were used, the protein being either labelled, e.g. by fluorescence markers, or detected by typical immunological methods. The laborious procedure for the derivatization of DNA with biotin has been simplified by introducing a photo-activatable analog of biotin for the labelling of nucleic acids. However, the number of biotin groups which can be introduced to the DNA molecule, is limited in both methods. It has been pointed out that the technique is extremely difficult to carry out successfully and the results obtained are quite varied. The method is therefore extremely unreliable and does not provide any basis for routine polynucleotide sequence detection. Therefore, the approach of cross-linking biotin labelled histone to single stranded nucleic acids with glutaraldehyde, was very interesting. Yet, despite the possibility of a substantial increase in the biotin content, the sensitivity of assay was not sufficiently high.

Some general remarks on known labelling techniques

It is obvious that in the design of hybridization probes all labelling methods based on random derivatization of the exocyclic amino function in nucleic acids with an appropriate label must employ polydeoxy nucleic acid. The short DNA sequence for example oligo is too sensitive for such an operation if

effective further hybridization is expected. The use of a long DNA probe implicates that the hybridization temperature has to be relatively high unless special additives e.g. a high concentration of DMF, are present in the test mixture. Such a high temperature may limit the use of some enzymes as detectable markers by decreasing their activity. Moreover, it is well known that a longer sequence requires a much longer time for hybridization to proceed, a fact which seriously limits the speed of assays without giving a proportionally equal increase in sensitivity.

In the literature there are only few reports of employing oligodeoxynucleotides labelled with non-radioactive markers for use as hybridization probes. The use of octadecamer selectively monobiotinylated at the 5' position was not successful while the sensitivity of the assay was far too low. It is therefore commonly accepted that in all methods making use of indirect labelling or indirect detectable markers, the preferred procedure involves amplification of the signal. This is laborious, increases errors of method and makes routine analyses very difficult.

Polymeric substances of the non-nucleotide type have already been employed in the design of non-radioactive hybridization probes. Biotin-labelled histone has been cross-linked to the single-stranded nucleic acids with glutaraldehyde but the detection of target DNA with these probes and avidine-peroxidase conjugates was less sensitive than the radioactive methods. In another strategy, peroxidase or alkaline phosphatase was cross-linked to polyethyleneimine of low molecular weight with p-bensoquinone and the resulting conjugates were cross-linked to DNA with glutaraldehyde. In both cases the polymers were used mainly as linkers between the DNA probe and the detectable units. It is therefore important to underline that in our approach the main object of using a polymeric matrix is to amplify the detectable signal thus making the assay more sensitive, and not to use it as a simple linker molecule.

Hybridization assays employing rare earth chelates have been described before our priority date (EP-A-97,373; Syvänen et al, Nucl Acids Res 14(1986)1017-27; and Hurskainen et al, In: NOMBA Nordforsk Symposium Gene Technology in Basic and Applied Research (Abstr.) Savonalinna, Finland, May 27-29, 1984, 12) Further details have also been given during the priority year (WO-A-87/02708; and EP-A-212,951)

A survey of the literature clearly shows that if the aim is to design an easy, reliable and sensitive hybridization method, it is desirable to use directly labelled and detectable probes, and, in contrast to sandwich techniques, when amplification of signal is required it should be realized by increased label density. The method of choice should not involve either radioactive isotopes or highly toxic intermediates.

The invention

The present invention provides an improved hybridization assay method utilizing certain labelled nucleic acid probes. It is a method for the detection of a nucleotide sequence of a nucleic acid in a sample. The method has the following major characteristic steps:

(i) Contacting under hybridization condition the single stranded form of the nucleotide sequence present in a sample with a single stranded nucleic acid probe, so as to form a double stranded nucleic acid that as one of its strands has the nucleotide sequence to be detected and as the other strand the nucleotide sequence of the probe. The probe has as one part a nucleotide sequence that is complementary to the sequence to be determined and as the other a plurality of rare earth metal chelate groups that are covalently linked, via a water-soluble polymer of non-nucleic acid structure, to its nucleotide sequence. One probe can contain many sequences complementary to the one to be determined and/or of pluralities of rare earth metal chelate groups.

(ii) Determining or detecting the double stranded nucleic acid so formed by using time-resolved spectrofluorometry to measure the rare earth metal chelate incorporated into said double stranded nucleic acid

Above the plurality of rare earth metal chelate groups has at least one metal ion selected from the group consisting of Eu^{3+}, Sm^{3+}, Tb^{3+} and Dy^{3+}, preferably the europium and terbium ions. The intensity of fluorescence emitted from the double stranded nucleic acid is a quantitative measure of the nucleotide sequence to be determined.

The use of covalently bound polymeric group carrying several rare earth metal

chelate groups amplifies the signal from the probe. A brief calculation of the fluorescence intensity using the probe labelled with only a few fluorescent markers showed that the sensitivity of such an assay will be insufficient. This is especially valid in the detection of viral DNA in the early stages of infection. Therefore, the important feature in the invention is the use of water soluble polymeric compounds as a matrix to which a large number of europium or terbium chelates are covalently coupled. This is equivalent to the large amplification of the detectable signal, but contrary to many existing methodologies, the present one is a straightforward, direct, and one-step procedure.

One aspect of the invention is the probes that are described in this specification and that are used in our invention. From practical considerations and as shown in the examples, the chelated lanthanide ion is always non-radioactive.

Hybridization assays

Hybridization assays are well-known in the art. As in common immunoassay techniques they can be divided into homogenous and heterogenous ones. The homogenous hybridization assays utilize labels that in one way or the other change their signal as a consequence of being incorporated into double stranded forms of DNA. Accordingly no separation of single stranded from double stranded nucleic acids is required in the homogenous variants (see for instance EP-A-144,914 and EP-A-70,685). In the heterogenous ones a separation is accomplished by using a matrix that is insoluble in the assay medium and which selectively can bind either probe-containing double stranded nucleic acids from single stranded probes or vice versa. The binding may be carried out as a biospecific absorption employing covalently bound oligonucleotide sequences or other biospecific affinity reactants. Isolubilised streptavidin or antibodies can be caused to specifically absorb nucleic acids equipped with biotinyl or homologous haptenic groups, respectively. (See for instance Dunn et al, Cell 12 (1977) 23-36; Ranki M et al, Gene 21 (1983) 77-85; Meinkoth and Wahl, Anal Biochem 138 (1984) 267-84; Syvänen et al, Nucl Acids Res 14 (1986) 5037-48; Dattagupta and Crothers, EP-A-130,523; WO-A-85/02628 and US-A-4,563,419). The most popular heterogenous method at present time is hybridization on filter paper, e.g. nitro cellulose paper. In this latter method the single stranded DNA to be assayed is adsorbed to the filter paper, whereafter the filter paper is saturated with DNA non-homologous to the one to be assayed and contacted under hybridization condition with the DNA probe.

The condition required to accomplish hybridization depends on the probe length, i.e. the length of the oligonucleotide sequence to be hybridized, and the specificity desired. As a general rule longer nucleotide sequences that are complementary require a longer time for hybridization to occur, although an increase in temperature and/or special additatives, such as DMF, may give more rapid reactions. Normally the hybridization solutions are buffered to pH values of 5-9 and the hybridizations are carried out at constant temperatures, 18-65°C for 3-48 hours. For homogenous variants it is very critical not to add to the hybridization media chemicals negatively influencing the signal emitted by the label(s).

The polymer and its derivatization

The water-soluble polymer is of non-nucleic acid structure, and may be a biopolymer or a synthetic polymer. Among the former derivatized forms of biopolymers are included . When non-bound to a nucleic acid the polymer to be chosen exhibits a plurality of functional groups allowing covalent attachment to a nucleic acid. Thus the most suitable polymers have more than 10, such as more than 50 OH-groups or amino groups. The OH-groups may be part of a carboxylic acid group or a alcoholic or phenolic hydroxyl group. The polymer can have molecular weights above 1,000 such as above 5,000 dalton. In most cases the molecular weight is below 10^6 dalton.

Linear or substantially linear polymers are preferred because of the favourable geometry of the hypothetic hybrids. An other obvious demand placed on such polymers is their solubility in water. Free water solubility of their chelate derivatives, as well as the water solubility of the final lanthanide chelate-polymer-DNA complex are imperative.

The first successful attempt to synthesize derived polymers was made by condensation of polyacids with chelates possesing a free amino group, utilizing a water soluble carbodiimide as the coupling reagent. Next, since most of the chelates available were best suited for derivatization of free, preferably primary, amino groups with relatively high pKa (optimum region pKa 8 - 11), polymers which pass such functions were directly tested. Other polymers of interest were prepared by appropriate derivatization, introducing free amino functions to the polymers which lack them from the beginning.

Particularly good coupling results were obtained with the following polymers: chemically modified dextrane, polyvinylamine (PVA), polyethyleneimine (PEA), polylysine (PL), chemically modified polyacrylamide, carboxymetylated polyvinylamine (CM PVA) and polyacrylic acid. Two of these, polyethyleneimine and dextrane, do not fully meet the criterion of linearity. Nevertheless, both compounds consist of long intervals within which the required linear geometry is preserved and accordingly are substantially linear. All these polymers could be readily dissolved in water at all derivative stages.

Polyvinylamine was prepared according to a known method, and stored in a convenient form of hydrochloride as dry powder. This material (M^{PS} 3.4 x 10^4) has been used as a matrix for the synthesis of polymeric water soluble dyes and has been shown to be the best alternative owing to its high reactivity and the almost highest density of the groups which can be derivatized. Polyethyleneimine used in our experiments had an average molecular weight of 5 x 10^4 to 6 x 10^4, 1,5 x 10^4 to 3 x 10^4, and polylysine hydrobromides 3 x 10^4 to 7 x 10^4, respectively. Functionalized polyacrylamide was obtained in our laboratory from polyethylacrylate Mw 72,000. The synthesis is described as Example 1. The polyacrylic acid used had a Mw ≈ 5,000. The procedure for carboxymethylation of polyvinylamine is outlined as Example 2.

Polyacrylamide is an example of an approach where the desired compound is prepared not by derivatization of already existing polymeric material but through the synthesis of a monomeric acrylamide chelate and its subsequent polymerization (Example 3). Copolymerization of such monomers with other appropriate acrylamide derivatives will create products possessing all the necessary features like proper solubility, net charge, and the presence of other functional groups.

Theoretically, two different derivatization methods are possible for polymers possessing free amino functions. We found that derivatization with a lanthanide chelate followed by a derivatization with an appropriate bifunctional reagent, and coupling of the activated oligo-DNA or poly-DNA probe is the more economical one. Reverse order of the reactions is also possible since the reactivity of the exocyclic amino function in DNA is very low, and thus no derivatization with chelates takes place on these amino functions. This has

been verified in several blank reactions with DNA and the active forms of chelates.

For acidic polymers (polymers containing for instance carboxylic acid groups) only the first route, i.e. functionalization with a chelate, followed by reaction with a bifunctional coupling reagent and the addition of nucleic acid could be realized.

Both in case of polyamines and polyacids polymers, the reaction cycle could be stopped at the level of an activated polychelate (Examples 4 and 5). Such functionalized polymers could be stored for long period of time and coupled with different DNA probes whenever necessary. This type of derivatization has therefore the advantage of being "universal".

Functionalization of acidic polymers has been found to be a very quick process, and the degree of condensation, performed in a water solution and employing a large excess of EDAC (water-soluble condensing agent), was directly proportional to the amount of used amino reagent.

The labelling of amino-functionalized polymers with chelates has been performed in aqueous media using a triethylammoniumbicarbonate buffer, pH 10.

The use of a phosphate buffer should be avoided as it forms unsoluble salts in water with polyamines. The extent of the derivatization could easily be manipulated by changing the pH or the chelate concentration. For instance, using two equivalents of isothiocyano chelate per each amino group in PVA at pH 10, essentially all amino functions could be derivatized in an overnight reaction.

By performing this reaction at pH 7 only 35-50% of the amino groups were labelled. This was determined after standard gel filtration and counting of lanthanide fluorescence.

The nucleic acid part of the probe

Two general alternatives exist in the choice of the nucleic acid part of the probe.

The use of polynucleotides is often favourable when the nucleic acid is easily accessable and no data exist on the sequence of the target molecule. The probe could be prepared e.g. from a double-stranded DNA identical in sequence to two strands of the gene sequence to be detected. On denaturation of the double-stranded DNA the two strands would be hybridized to two strands of the gene sequence to be detected. The probing sequence of its double-stranded precursor can be produced by cloning it in a plasmid or a phage. Multiple labelling of such poly-DNA probes with polymeric lanthanide chelates will therefore be an attractive procedure making it possible to overcome the disadvantages connected with labelling and amplifying the signal as in cases where other non-radioactive markers are used.

The native molecule of a nucleic acid has to be modified by introducing groups which are able to selectively react with a potential bifunctional coupling reagent. An example of a group which fulfils these criteria is the thiol group and it can be introduced by employing several existing procedures (Example 6). This direct procedure is rapid, reliable and safe and should allow inexpensive small or large scale labelling of any DNA with lanthanide chelates.

The already mentioned low reactivity of natural amino functions in DNA permits only slight cross-linking between DNA and polymeric chelates. This is necessary for persistance of the intact fragments in DNA which are vital features of efficient hybridization. Nowadays there is, however, a tendency to employ short oligonucleotides instead of longer poly-DNA fragments as hybridization probes. This is especially important in the design of routine assays when time plays an important role. These small fragments with the sequence long enough to be specific for the sequence to be detected should theoretically contain at least 16 nucleotides. Such oligonucleotides can today be easily synthetized by employing commercially available reagents and apparatus, even by non-chemists. An additional feature of the synthetic DNA fragments is the possibility of their specific and regioselective derivation at protected state. One of these reactions is the recently published procedure for selective 5'-thiolation of synthetic oligonucleotides (Example 7). This, in conjunction with the already existing procedures of the specific terminal derivatization even in a fully deprotected state, together with the simple preparative purification methods make them an interesting alternative for the polydeoxynucleatide probes

(Examples 8 and 9). However, perhaps the greatest advantage of short DNA fragments becomes clear when considering the hybridization process.

The oligo DNA probes characterized by the very favourable kinetics of hybridization allowing the assay to proceed at low temperature and in a much shorter time period than those which are necessary for poly-DNA probes. This is of course, only true of relatively free oligo-DNA probes. The ones which are bound to globular molecules e.g. proteins may behave very differently and in the most drastic case even totally loose the basic pairing properties. Fortunately, however, the cross-linking of 5'-functionalized oligo-DNA probes to the linear polymers was successful without any detectable differences in hybridization efficiency as compared to free probes.

Rare earth chelates

Different types of functionalized rare earth chelates are known from the art. Some of them exhibit fluorescence when excited at the appropriate wave length. Others do not. The fluorescent property is not critical for their use in heterogenous variants of our invention, because techniques have been developed that can transform non-fluorescent rare earth chelates to fluorescent ones. (Hemmilä et al., Anal.Biochem. 137 (1984) 335-43). For homogenous variants, it is more critical to select chelate having fluorescent properties. It is thus more important to select the chelate according to the stability that is required in the hybridization assay than to have inherent fluorescent properties as the criteria.

In order to determine if a given chelate has the satisfactory stability, it is simply tested in the assay to be run. If the sensitivity is satisfactory the chelate is of satisfactory stability. Suitable chelates to be used in our invention mostly exhibit carboxylate, phosphate or phosphonate anionic groups and/or primary, secondary or tertiary amino nitrogen atoms so located in the molecule that they simultaneously coordinate, via their negatively charged oxygen or nitrogen, respectively, to the rare earth metal ion so that more than three, preferably more than four, five- or six-membered rings are formed. This definition means that the chelates in question have more than four, preferably more than five heteroatoms selected among said nitrogens and oxygens, and that the rare earth metal ion is a joint member for all the rings. Nitro-

gens in aromatic rings are included in tertiary nitrogen atoms. Five-membered rings are preferred. This definition of a stable chelate can be found in common text-books and covers those given in the patent literature given below.

Different types of chelates that can be used in our invention have been described previously (EP-A-195,413; EP-A-139,675; EP-A-68,875; EP-A-203,047; EP-A-171,978; EP-A-2,570,703; US-A-4,352,751 and US-A-3,994,466). With respect to hybridization assays that require extremely harsh conditions we have developed a series of chelates that is represented in example 10. They have as the common denominator a pyridine ring substituted at the 2 and 6 positions with groups that together with the pyridine nitrogen can chelate a metal ion. To the pyridine ring is only bound hydrogens and/or aliphatic carbon atoms. These extremely stable chelates comply with the definition for stable chelates given in text-books.

The chelate employed has to be sufficiently stable under the assay steps requiring harsh conditions, e.g. elevated temperatures (above 60°C), presence of other chelating agents (EDTA etc.) etc.

To each linear polymer nolycule may have been bound more than 10, such as more than 25 chelating groups. Based on the total amount of lanthanide chelate groups in a given lanthanide chelate polymer and the funcional groups potential of use for introducing the same group the substitution degree of the lanthanide chelate polymer is usually more than 20% and can in may cases also exceed 50%.

EXAMPLE 1: Preparation of amino functionalized polyacrylamide (Scheme 1)

2,0 g of polyethyl acrylate (M.w. 72000 - Aldrich) was treated at 50°C with 50 ml of dry ethylenediamine in a 100 ml round bottom flask using a slow speed magnetical stirrer. After 24 h stirring the mixture was evaporated to dryness on a rotavapor using an oil pump, and coevaporated three times with n-butanol. The residue after dissolving in 10 ml of methanol was acidified with 5 M HCl and again evaporated to dryness. The solid crude product was dissolved in water (20 ml) and precipitated from acetone.

Elementary analysis of the material showed a minimum of 80% conversion of starting ester functions.

EXAMPLE 2: Preparation of carboxymethylated polyvinylamine (CMPVA) (Scheme 2)

1,0 (12,6 mmol) of polyvinylamine hydrochloride (PVA HCl) was dissolved in 20

ml of water and the pH of solution was brought up to 10.5 by addition of 5 M NaOH. 10.5 g of bromocetic acid (75.6 mmol) dissolved in 20 ml of water and neutralized with NaOH was dropped into magnetically stirred PVA solution, maintaining pH 10.5 by addition of 5 M NaOH. After complete addition the mixture was left stirred overnight. The crude product was isolated by addition of 500 ml of ethanol. The white precipitate was separated, dissolved in water and dialyzed against distilled water.

EXAMPLE 3: Preparation of functionalyzed polychelates by the polymerization of monomeric units (Scheme 3 and 4)

A) Synthesis of acrylamido chelate (scheme 3). 100 mg of an amino functionalized lanthanide chelate (EP-A-139,675) was dissolved in 5 ml of 1 M trimethylammonium bicarbonate buffer pH 9.5 and cooled to 0°C. To this stirred solution 1.5 ml of acryloyl chloride was injected in few portions. Progress of the reaction can be monitored on TLC using acetonitride - H_2O (4:1) as solvent. The mixture was stirred for 1 hr and evaporated to dryness on a rotavapor, followed by several coevaporations with water. The final residue was dissolved in a small amount of water and lyophilized in high vacuum.

AA) Analogously the chelates of example 10 (compounds 12) can be used.

B) Synthesis of monoacylamido derivative of diaminoalkane - general description (scheme 4). Monotrifluoroacetate of a (α,ω) diaminoalkane was dissolved in dry pyridine: dichlorometane (1:1) and cooled to -10°C. To the stirred mixture 1.5 ml acryloyl chloride was added. The mixture was stirred for 30 min. and partitioned between $CHCl_3$ and saturated $NaHCO_3$. The organic extracts were evaporated and coevaporated three times with toluene. The oily residue was dissolved in methanol (10 ml/mmol) and an equal volume of saturated Na_2CO_3 was added at once. The turbide mixture becomes clear after about 1 hr and the hydrolysis of the trifluoroacetamido group was virtually complete after 5 h. The reaction mixture was then evaporated to a small volume and extracted six times with $CHCl_3$/EtOH (1:1) after addition of saturated $(NH_4)_2SO_4$. After evaporation the extract was purified by short column chromatography using $CHCl_3$/EtOH (6:4) as the final solvent. After evaporation of proper fractions the oily product was stored at -20°C after addition of a polymerization inhibitor.

C) Method for synthesis of functionalized polymeric chelates. To a mixture consisting of acrylamido chelate (point A), functionalized acrylamide (point B), in a ratio of 2:1 and dissolved in phosphate buffer (μ = 0.01) pH 8,0, so that the concentration of acrylamido monomers was 5%, TEMED (5% aqueous solution) and ammonium persulfate (5% aqueous solution) were added to a final concentration of 0.047% (TEMED) and 0.033% (ammonium persulfate). The mixture was kept at 50°C for 30 min, and the polymer was separated from low molecular weight components by gel chromatography.

When using this method, polymers with chelate functions at up to 60% of its amido groups could be easily obtained. By also including other acryl monomers, e.g. acrylated amino acids or acrylated taurine and by varying the ratios of added monomers polymers of different constitution could be synthesized.

CC) By using the acrylamido chelate of step AA other functionalized polyacrylamides containing the corresponding chelate can be obtained.

Example 4: Application of carboxymethylated polyvinylamine (CMPVA) for the synthesis of activated polymeric chelate (scheme 5)

5.85 mg (0.01 mmol) of Eu^{3+} chelate in aminoform (same as in Example 3A) was dissolved in 50 µl of water. To this solution a solution of 2.0 mg (0.02 mmol - based on carboxylate) carboxymethylated polyvinylamine in 30 µl of water was added. Solid EDAC (38 mg - 0.20 mmol) was then added in three portions during 1 hr, and the pH of the solution was maintained at 5.5 by addition of diluted HCl. To this reaction mixture a bifunctional reagent (see scheme 5), (0.32 mg - 0.001 mmol), was added, followed by EDAC (19 mg - 0.01 mmol). The mixture was incubated for 30 min, and the product was precipitated by addition of acetone. The solid, centrifugated material was dissolved in water and the polymeric product was separated by filtration through a Trisacryl column (2 x 50 cm) using 50 mM Tris-HCl, 0.5 NaCl buffer pH 7.0.

The determination of the europium content in pooled high molecular fractions established a 60% incorporation of the starting monomeric chelate, which corresponds approximately to 200 chelates per polymeric molecule. Finally, the material was dialyzed against water, precipitated from acetone and stored as a dry powder.

EXAMPLE 5: Application of polyvinylamine for the synthesis of activated polymeric chelate (Scheme 6)

Step A. 500 µg (6.3 µmole based on amino function) of polyvinylamine hydrochloride, dissolved in 20 ml of H_2O, was labelled with 10 µmoles of an isothiocyanate functionalized lanthanide chelate (see EP-A-139,675 and compound 13 of Example 10) in the presence of 10 µmoles of triethylamine. The reaction mixture was incubated overnight at 20°C. The unreacted chelate was removed by gel filtration through a Sephadex^R G-50 column (0.7 x 20 cm). Fractions containing lanthanide ions were pooled, desalted and concentrated. This procedure gave a polymer with a high degree (50-75%) of substitution.

Step B. The polymeric chelate (from step A) was dissolved in 50 µl of phosphate buffer pH 6.5 and a bifunctional coupling reagent (see scheme 6), 270 micrograms (0.63 micromol) in 10 µl of ethanol, was added. The reaction mixture was incubated for 6 hr with occasional shaking. The product was isolated after filtration through a Sephadex^R G-50 column and precipitation from acetone.

EXAMPLE 6: Construction of lanthanide labelled polydeoxyribonucleic acid

Variant A:

10 µg of single-stranded phage M 13 mp 10 DNA containing an Adenovirus 2 Xba I restriction fragment (0-3.85 arbitrary units) of about 1350 bp is reacted with 150 µl of 25% glutardialdehyde and 50 µg of an europium-labelled PVA (from Example 5) in phosphate-buffered saline (10 mM Na-K-phosphate pH 7.4, 0.18 M sodium chloride). The reaction is allowed to proceed for 20 minutes at 37°C. After incubation the non-reacted aldehyde groups were blocked by reacting with 500 µl of 1 M lysine, pH 7.5 at 20°C for one hour.

Variant B

Step A: 200 µg of single-stranded M 13 DNA containing a fragment of Adenovirus 2 DNA was modified with sodium bisulfite-ethylenediamine at pH 6.5 for 2 h according to published methodology. The dialyzed and concentrated sample was then dissolved in 1 ml of PBS (pH 8.5). Solid S-acetylmercaptosuccinic anhydride (1 mg - 5.75 µmol) was added and the reaction mixture was kept at R.T. for 60 min with occasional shaking. The mixture was made 0.3 M with respect to

sodium acetate and 2.5 volumes of ethanol was added to precipitate DNA. The precipitate was dissolved in 1.0 ml of 0.01 M sodium hydroxide and kept for 60 min at R.T. for hydrolysis of S-acetyl protecting groups after which the mixture was made neutral with 0.1 M HCl. DNA was precipitated and purified from low-molecular weight compounds by gel chromatography through Sephadex^R G-25 (0.7 cm x 15 cm) in 0.05 M phosphate buffer, pH 6.5.

Step B: 200 µg of the purified single-stranded DNA containing free sulphydryl groups and 500 µg of Eu-PVA from example 4 or example 5, step B, were mixed and incubated at room temperature for 20 hours. The Eu-PVA-DNA conjugate was purified by gel filtration through a Sephadex^R G-150 column (0.7 cm x 47 cm). The column was equilibrated and eluted with PBS.

EXAMPLE 7: Construction of lanthanide-labelled oligodeoxribonucleic acid (Scheme 7)

Step A. A hexadecamer DNA probe was synthetized in large scale employing standard solution chemistry. The 5'-protecting group was then removed and the probe was labelled with a protected thiol function (S-Tr) according to the procedure of Connolly B.A., Nucl.Acids Res. 13 (1985) 4485-4502 modified by using phosphotriester chemistry, followed by standard deprotection and purification methods. Finally the S-Tr protecting group was removed with silver nitrate in buffered by sodium acetate system. The excess of silver was removed with hydrogen sulphide and the precipitated silver sulphide was filtered out with help of 2 µ filters. The clear filtrate was concentrated and desalted on Sephadex^R G-25 (10 x 200 mm).

Step B. 1.0 mg of 5'-thiolated oligo DNA probe was mixed with activated PVA chelate (from Ex. 5) at pH 7.0 and in different ratios (3:1 - 20:1). All mixtures were incubated overnight and separated using a Sephadex G-100 column (0.7 cm x 50 cm). The column was equilibrated and eluted with PBS.

EXAMPLE 8: Hybridization using DNA probe of long sequence

Step A: Adenovirus 2 DNA and PBR 322 as a control DNA were denatured and spotted onto nitrocellulose filters. Amounts of DNA from 100 ng down to 1 pg were applied. Filters were baked in a microwave oven for 3 minutes.

Step B: Filters obtained in Step A were prehybridised at 42°C for 2 hours in 50% formamide containing 1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.0, 5 x Denhardt's reagent (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin) and 50 µg per ml of denatured herring sperm DNA.

Step C: After prehybridization the filters were transferred to a hybridization solution containing 50% formamide, 1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.0, 5 x Denhardt's reagent, 0.5% sodium dodecyl sulphate (SDS) and 50 µg per ml of denatured herring sperm DNA. Eu-PVA-DNA conjugate (Examples 1, 2 and 3) was added to give the final probe a DNA concentration of 0.2 microgram/ ml. The filters were hybridized at 42°C for 4 hours. The filters were then washed at 42°C with 0.15 M NaCl, 10 mM Tris-HCl pH 7.0 containing 0.5% SDS three times for 15 minutes.

Step D: The spots in the filters were punched and the time-delayed fluorescence from europium in each spot were measured using enhancement solution (Wallac Oy, Finland). Sensitivity of the test is 10 pg of Adenovirus 2 DNA. Values which are twice the mean of the negative controls or more were considered positive.

EXAMPLE 9: Hybridization using oligomeric DNA probe

Step A: As in example 8.

Step B: Filters were presoaked at 30°C for an hour in 1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.0, 5 x Denhardt's reagent and 50 µg per ml of denatured herring sperm DNA.

Step C: After prehybridization the filters were transferred to a hybridization solution containing 1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.0, 0.5% SDS, 5 x Denhardt's reagent and 50 µg per ml of denatured herring sperm DNA. A Eu-PVA-hexadecamer conjugate from example 7, step B, was added to give a hexadecamer concentration of 20 ng/ml. Hybridization was carried out at 30°C for 3 hr. The filters were washed in 1 M NaCl containing 10 mM Tris-HCl pH 7.0 and 0.5% SDS first at 30°C for 15 minutes and then at 35°C for 10 minutes.

Step D: Spots were punched and the europium in each spot was measured as in example 8, step D. A positive signal was detected when the spots in the filters contained 200 pg or more of Adenovirus 2 DNA.

EXAMPLE 10: Synthesis of novel functionalized chelates

For a survey of the synthetic route employed and structures of the compounds involved see scheme 8. NMR-spectra were recorded for the end product and intermediates synthesized and found to be in accordance with the structures given. The compound numbers refer to those given in scheme 8.

Compound 2: Liquid ammonia (150 ml) was introduced to a 250 ml three-necked round bottomed flask equipped with a mechanical stirrer, dropping funnel and outlet tube and immersed in a dry ice/ethanol bath. Sodium amide was generated by addition of 20 mg of iron nitrate (Fe^{3+}) followed by metallic sodium (2.09 g, 0.09 mol). The deep blue solution was stirred for one hour and the solution of collidine (1) (10.06 g, 0.083 mol) in 20 ml of dry diethylether was introduced into the reaction -- addition time 15 min. The formed yellow suspension was stirred for an additional 45 min followed by the addition of benzylchloride (6.33 g, 0.05 mol) dissolved in 10 ml of dry diethylether. The reaction mixture was stirred for 45 min and the excess of sodium amide was neutralized by addition of ammonium chloride (4.82 g, 0.09 mol) dissolved in 20 ml of H_2O. Ammonia was evaporated and the residue was partitioned between water and diethylether. The collected etheral phase was dried over sodium sulphate and evaporated. The brown residual oil was fractionated collecting a fraction distilling at 130°C/0.1 mmHg. Yield = 6.17 g (55%), oil.

Compound 3: Compound (2) (20 g, 88.9 mmols) was dissolved in THF (150 ml), and nitric acid (6.7 ml, 60% aq. solution, 1 eq) was added. Diethylether was added to the clear solution until it remained dimmy, and the mixture was left in the freezer for crystallization. The white crystals of nitrate (quantit. yield) were added in small portions to well-chilled sulphuric acid (150 ml) never allowing the temperature to reach 10°C, whereafter the mixture was warmed at 50°C for 10 min. The resulting brown solution was poured onto ice and neutralized with solid sodium hydrogen carbonate. The organic material was extracted with chloroform (3x200 ml), and after drying over sodium sulphate, the

chloroform extract was flash chromatographed using 4% ethanol/chloroform as a solvent. The appropriate fractions were collected and evaporated yielding a pure yellow solid. Yield: 22.82 g (95%).

Compound 4: Compound (3) (22.82 g, 84.5 mmol) was dissolved in chloroform (100 ml), and 16 g (94 mmol) of m-chloroperbenzoic acid (mCPBA) was added in small portions at RT over a period of 30 min. The mixture was stirred for an additional 2 h and after a negative TLC test for the starting material it was worked up by partitioning between sat. sodium hydrogen carbonate and chloroform. The combined chloroform extracts (3x200 ml) were evaporated yielding a light yellow solid material that was TLC pure. Yield: 24.55 g (100%).

Compound 5: Compound (4) (24.0 g) was suspended in 100 ml of acetic anhydride. The mixture was refluxed for 20 min which resulted in a homogenous dark solution. Acetic anhydride was evaporated on a Rotavapor and the oily residue was neutralized with saturated sodium hydrogen carbonate, followed by extraction with chloroform (3x200 ml). The chloroform phase was evaporated and the crude material was flash chromatographed using 2% ethanol/chloroform as a solvent. The pure fractions were evaporated yielding oil that was TLC and NMR pure. Yield: 19.55 g (69%).

Compound 6: Compound (5) (19 g, 60.5 mmol), was oxidized as described in Example 3. The crude, single spot on the TLC product was isolated after standard work up. Yield: 18.97 g (95%), oil.

Compound 7: Compound (6) (18.5 g, 56 mmol), was converted to product (7) in a synthesis analogous to the synthesis in Example 4. The neutralized, end-extracted product was evaporated and flash chromatographed using 2% ethanol/chloroform as a solvent. The pure fractions containing the product were combined and evaporated. Yield: 12.04 g (61%), oil.

Compound 8: Diacetate (7) (12.0 g, 34.1 mmol), was dissolved in 50 ml of ethanol. To this solution stirred at RT, sodium hydroxide 5 M, 20 ml was added at once. After 10 min, when the TLC test for the substrate was negative, the mixture was neutralized with citric acid, and partitioned between sat. sodium hydrogen carbonate and ethanol/chloroform 1:1. The extraction was repeated three times using 100 ml of organic solvent for each extraction. The combined

extracts were evaporated and the residual mixture was flash chromatographed using finally 8% ethanol/chloroform as a solvent. The appropriate pure fractions were collected and evaporated. Yield: 4.75 g (52%), yellow solid.

Compound 9: To the dihydroxy compound (8) (2.7 g, 9.44 mmol), in 35 ml of dry dichloromethane, phosphorus tribromide (3.63 g, 1.26 ml, 13.41 mmol) was added and the mixture was refluxed for 15 min. The reaction mixture was neutralized with saturated sodium hydrogen carbonate and extracted with chloroform (3x50 ml). The combined extracts were concentrated and crystallized from ethyl acetate. Yield: 3.91 g (84%) - white crystals.

Compound 10: Compound (9) (3.27 g, 7.9 mmol) and iminodiacetic acid diethylester (5.78 g, 30.5 mmol), were coevaporated together with toluene and redissolved in dry acetonitrile (50 ml). Solid sodium carbonate (10 g) was added and the mixture was refluxed for 2 h, whereafter the salts were filtered out and the filtrate was evaporated. The residue was flash chromatographed and the fractions containing the product evaporated to dryness. To achieve material free from any co-chromatographed iminodiacetic acid diethylester, the oily product was triturated with petrolether (3x20 ml) which yielded material free from any contaminations. Yield: 5.09 g (80%), oil.

Compound 12: To the solution of compound (10) (4.8 g, 7.5 mmol) in 50 ml of ethanol, 10% palladium on carbon (100 mg) was added followed by sodium borohydride (378 mg, 10 mmol). The reaction mixture was stirred at RT for 5 min and partitioned between sat. sodium hydrogen carbonate and chloroform. The chloroform extracts (3x50 ml) were concentrated and flash chromatographed to give the reduced form of compound (10) (= compound (11)) as an oil after evaporation. Yield: 3.89 g (85%).

The reduced form of compound (10) (250 mg) in 20 ml of ethanol, was treated with 1 M sodium hydroxide (10 ml) at RT for 3 h. The pure on TLC product (solvent system acetonitrile/water 4:1) was neutralized with 1 M hydrochloric acid and concentrated. To the residue dissolved in water (25 ml), europium chloride hexahydrate (60 mg) dissolved in 5 ml of water was added and the mixture was stirred for 30 min. The excess of europium salt was removed by raising the pH to 8.5 with saturated sodium carbonate solution and filtration of the precipitate. The clear solution was evaporated almost to dryness and (12) was

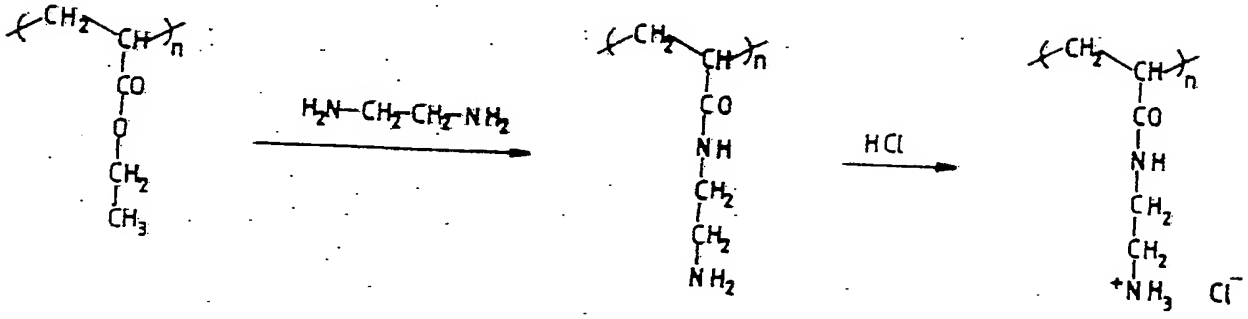
precipitated by addition of 100 ml of acetone. The product was washed on the filter with acetone and dried.

Compound 13: To the amino chelate (12) (100 mg) dissolved in 5 ml of water and vigorously stirred, tiophosgene (80 µl) dissolved in 3 ml of chloroform was added at once and the mixture was stirred at RT for 1 h.

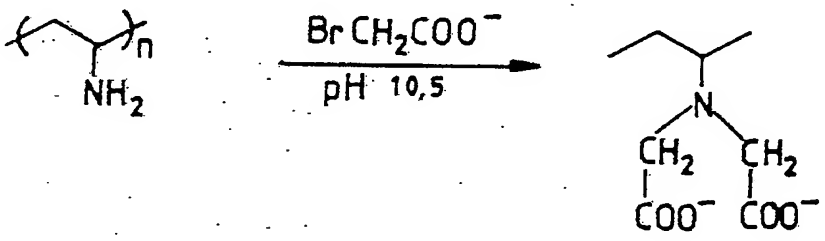
The water phase was separated, extracted with chloroform (3 x 3 ml) and concentrated to a volume of 0.5 ml. Addition of ethanol (10 ml) precipitated (13) quantitatively as white solid. The TLC (System Acetonitrile/H_2O 4:1) and fluorescence developing with acetonyl acetone/EtOH (1:20) showed only a single product being negative to a fluorescamine test for free amines.

Compounds 11 and 13 were employed in the preceeding examples.

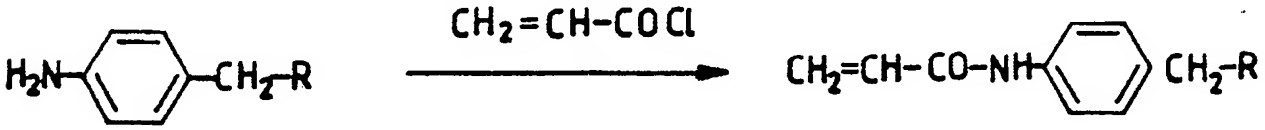
Preparation of amino functionalized polyacrylamide (Scheme 1)

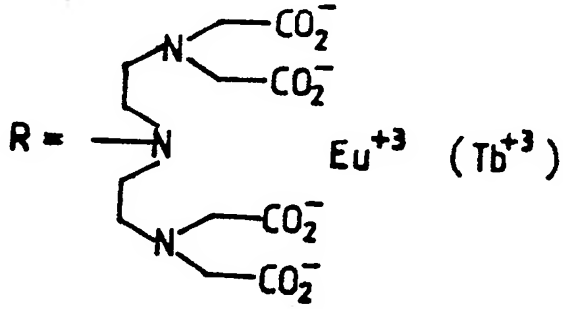


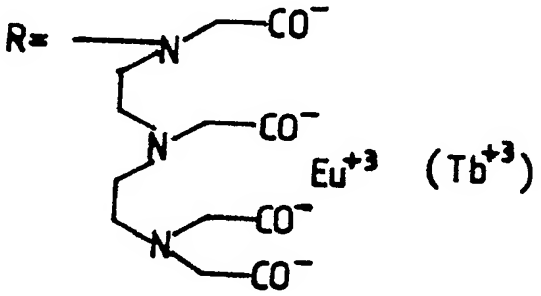
Preparation of carboxymethylated polyvinylamine (CMPVA) (Scheme 2)



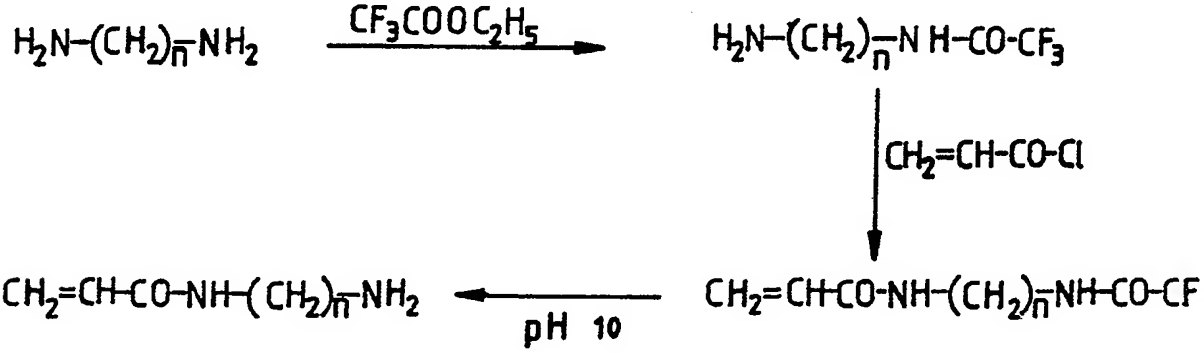
Preparation of functionalyzed polychelates by the polymerization of monomeric units (Scheme 3)



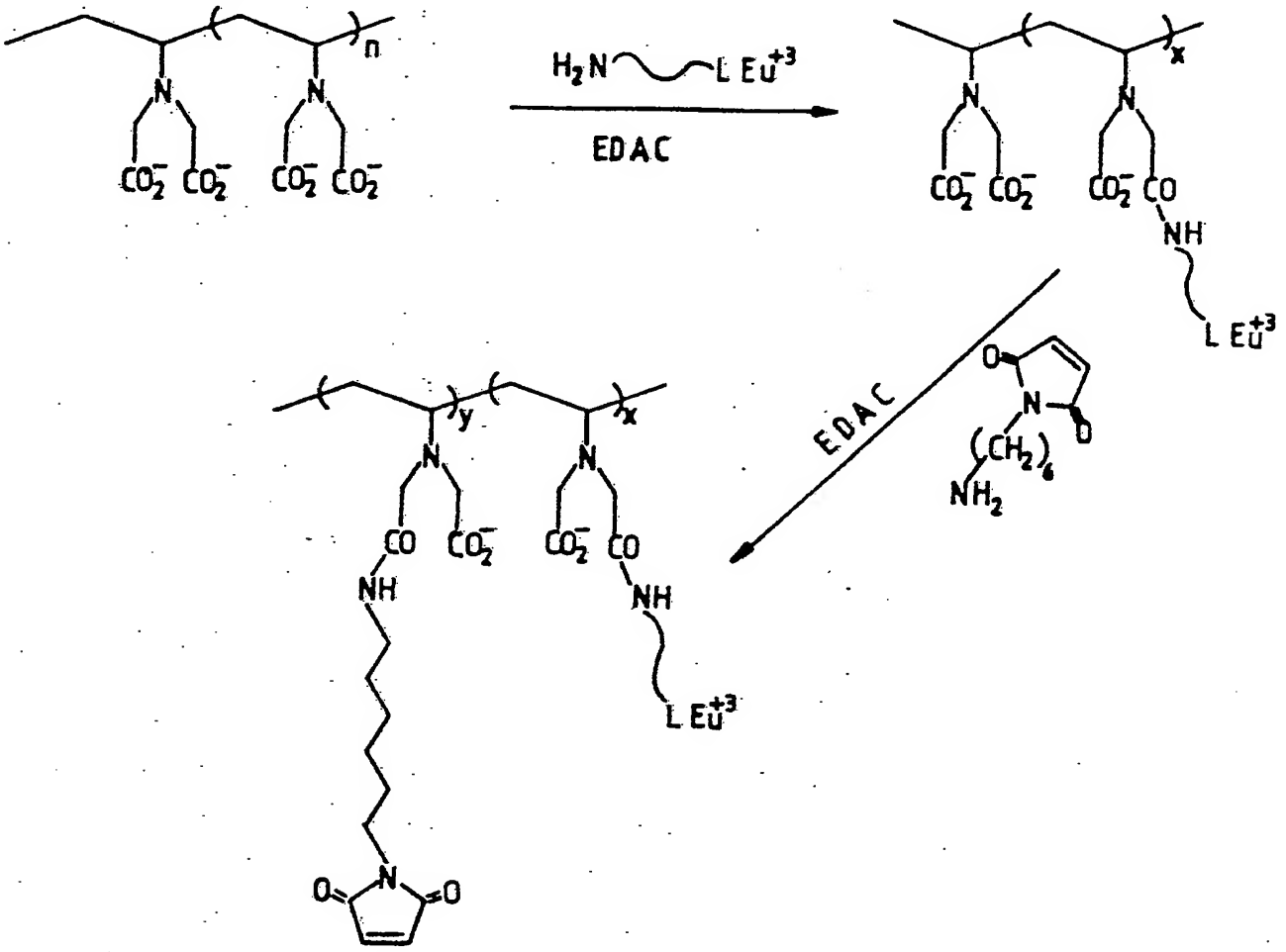




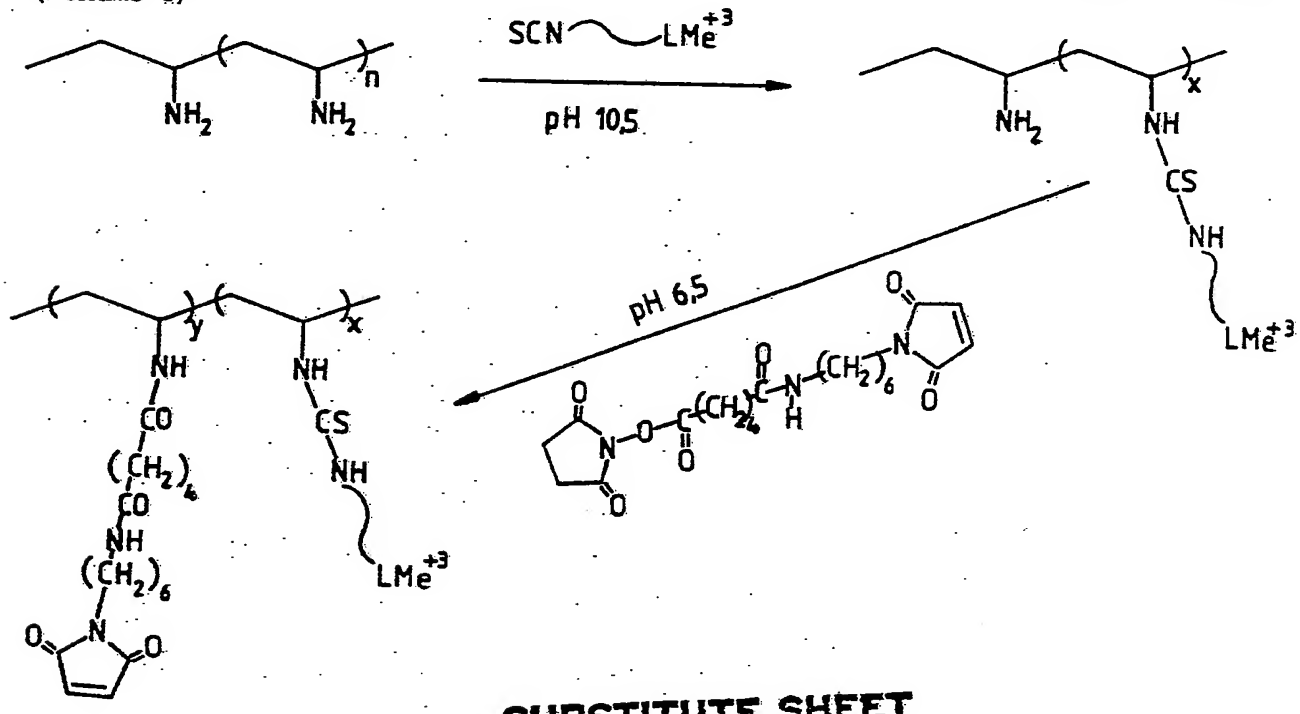
Synthesis of acrylamido chelates (Scheme 4)



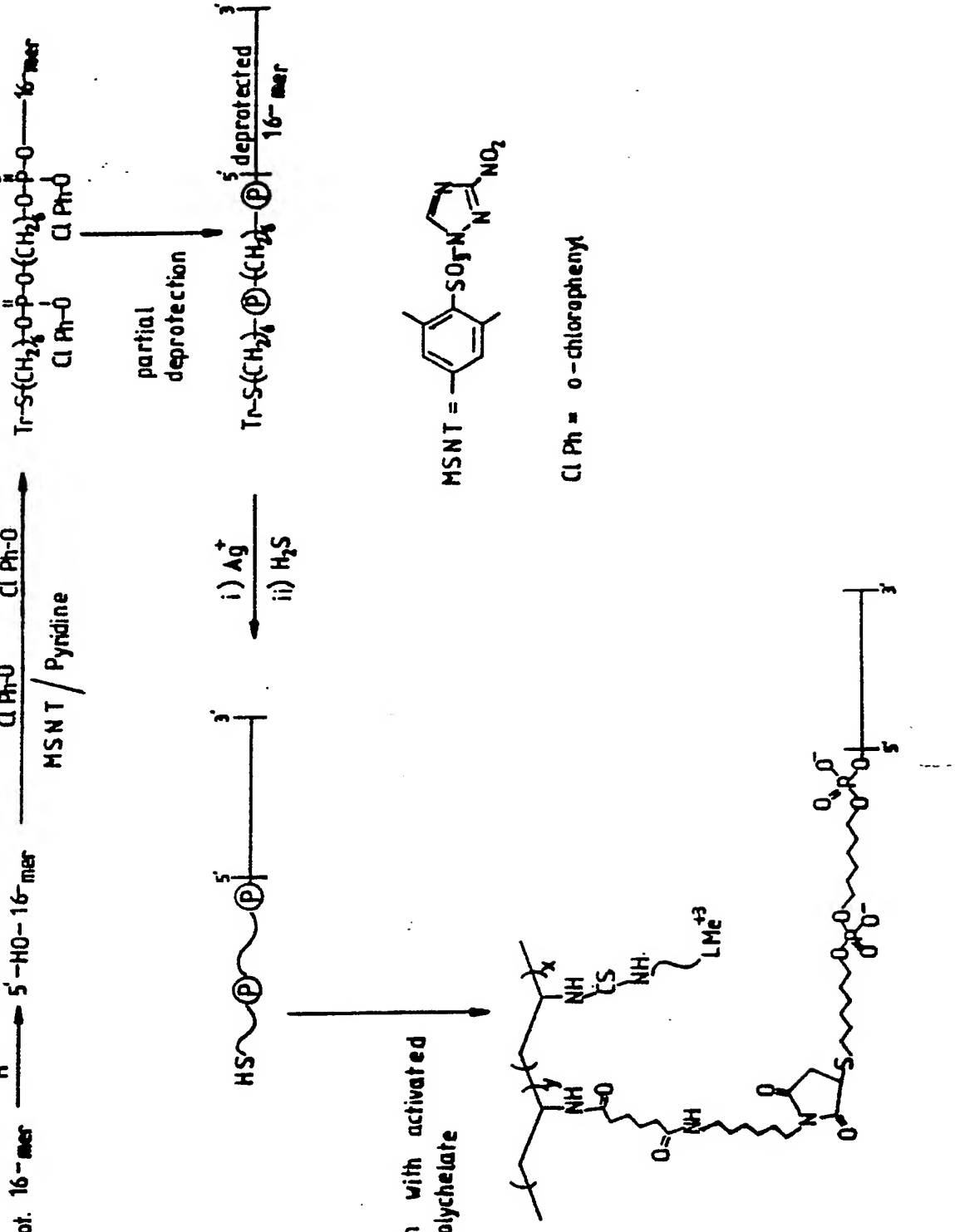
Synthesis of carboxymethylated polyvinylamine (CMPVA) having activated groups and chelate groups (Scheme 5)



Application of polyvinylamine for the synthesis of activated polymeric chelate (Scheme 6)

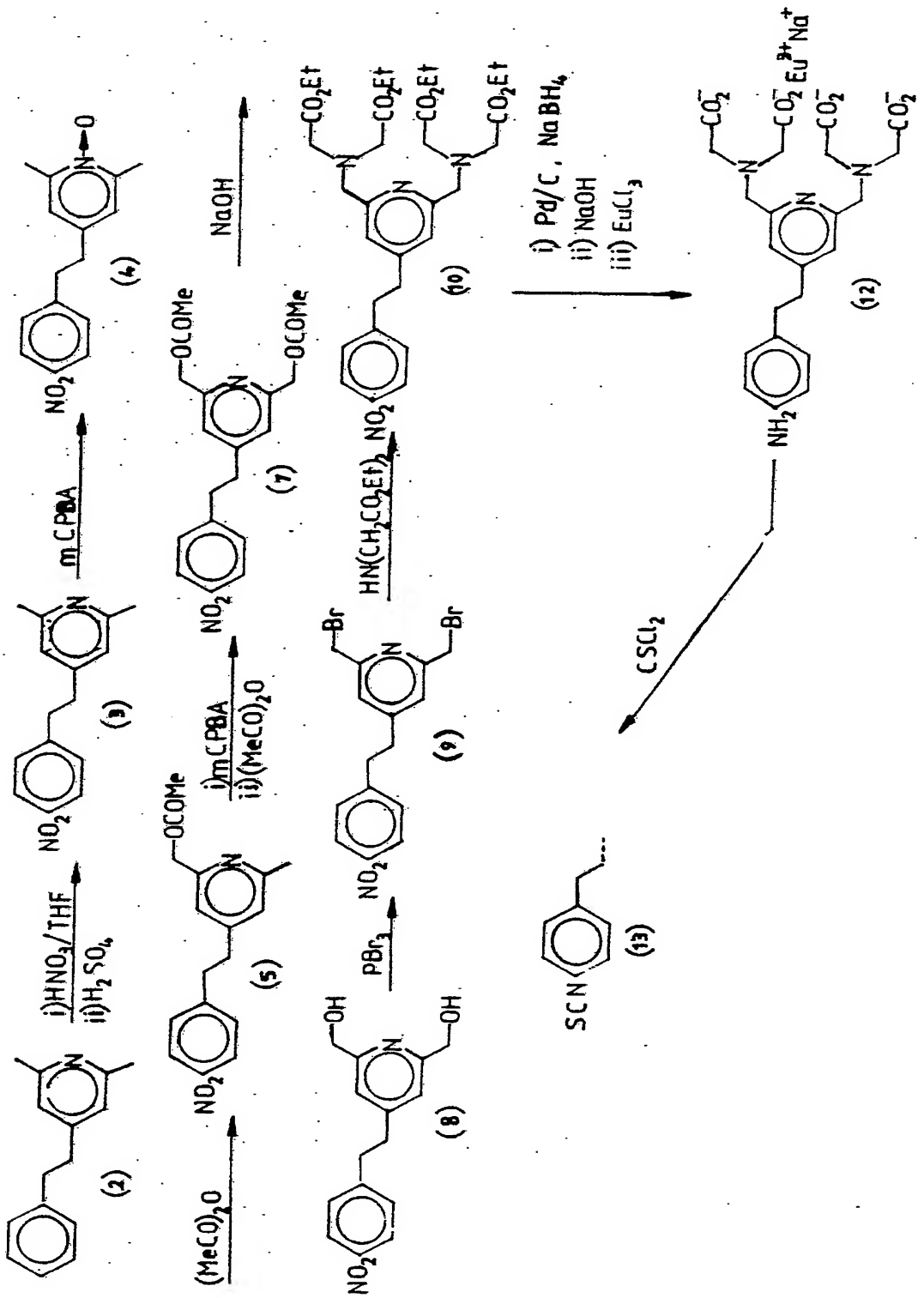


Construction of lanthanide-labelled oligodeoxribonucleic acid (Scheme 7)



Synthesis of new chelating compounds and chelates thereof

(Scheme 8)



Claims

1. A method for the detection of a nucleotide sequence of a nucleic acid in a sample, said method comprising the steps:

(i) contacting under hybridization condition the single stranded form of the nucleotide sequence with a single stranded nucleic acid probe, in which a plurality of rare earth metal chelate groups is covalently linked via a water-soluble polymer of non-nucleic acid structure to a nucleotide sequence complementary to the sequence to be detected, for the formation of a double stranded nucleic acid that as one of its strand has the nucleotide sequence to be detected and as the other strand the nucleotide sequence of the probe, and

(ii) detecting the formation of double stranded nucleic acid containing the probe by measuring the time-resolved fluorescence from the rare earth metal ion incorporated as a chelate in said double stranded nucleic acid.

said plurality of rare earth metal chelate groups having at least one metal ion selected from the group consisting of Eu^{3+}, Sm^{3+}, Tb^{3+} and Dy^{3+} as the chelated rare earth metal.

2. The method of claim 1, wherein the said at least one metal ion is selected from the group consisting of Eu^{3+} and Tb^{3+}.

3. The method of claim 1, wherein the water-soluble polymer is selected from the group of polymers having a plurality of OH- or amino groups.

4. The method of claim 3, wherein the water-soluble polymer is selected from the group of polymers exhibiting a plurality of OH groups, such as in polymers exhibiting a plurality of alcoholic, phenolic or carboxylic groups.

5. The method of claim 3, wherein the polymer is selected from the group of polymers consisting of polymers having a plurality of amino groups.

6. The method of claim 3, wherein the water-soluble polymer is selected from the group of polymers consisting of polyvinylamines, polyethyleneimines, polylysine, polysacharides, polyacrylamides, and derivatized forms of these polymers exhibiting the said plurality of OH- or amino groups.

7. The method of claim 1, wherein at least two nucleotide sequences complementary to the sequence to be detected are bound to the water-soluble polymer molecule.

8. The method of claim 1, wherein at least two water-soluble polymer molecules are bound to the nucleotide sequence complementary to the nucleotide sequence to be detected.

9. Nucleotide sequence to which a plurality of lanthanide chelate groups are bound covalently via a water-soluble polymer, said lanthanide being non-radioactive and selected from a group consisting of Dy^{3+}, Sm^{3+}, Eu^{3+} and Tb^{3+}, preferably from group Eu^{3+} and Tb^{3+}.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE87/00474

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC [4]

C 12 Q 1/68, G 01 N 33/58, 33/533, C 07 H 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC 4	C 12 Q 1/68, /00; G 01 N 33/53, /532, /533, /58
US Cl	435: 5, 6; 436: 500-504

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A2, 97 373 (ENZO BIOCHEM INC) 4 January 1984 p 1-8 and p 92-95 see in particular claims 17-19 and p 92, l 6-13 & AU, 16179/83 JP, 59062600 CA, 1223831	1-4
A	EP, A1, 84/03698 (LKB PRODUKTER AB) 27 September 1984 see p 2, l 14-35	1
A	EP, A1, 180 492 (COMMISARIAT A L'ENGERGIE ATOMIQUE) 1 May 1986 see p 22, l 16-20	1
A	Nucleic Acid Research vol 2, p 1017-28, published 1986 (Syvänen A-C et al) "Time-Resolved fluormetry: a sensetive method to quantify DNA-hybrids".	1
Y	EP, A1, 77 671 (ORTHO DIAGNOSTIC SYSTEMS INC) 27 April 1983 .../...	1-5

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1987-12-30	1988 -01- 13
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Carl Olof Gustafsson

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	JP, 58079162 US, 4434150 CA, 1200760	
A	EP, A1, 2 963 (EASTMAN KODAK CO) 11 July 1979	1-5
A	EP, A2, 195 624 (EASTMAN KODAK CO) 24 September 1986	1-5
A	EP, A2, 187 332 (MOLECULAR DIAGNOSTICS INC) 16 July 1986 see p 14-15 and claim 3	1-4